Chimeric transcription factors of this invention may contain, in addition to one or more copies of a p65 activation domain such as described above, one or more copies of one or more heterologous peptide sequences which potentiate the transcription activation potency of the transcription factor, as measured by any means. Inclusion of such motifs, including the so-called "glutamine-rich", "proline-rich" and "acidic" transcription activation motifs, in combination with a primary activation domain can result in extremely high levels of transcription.

A wide variety of transcription activation domains and motifs can be used in the practice of the present invention in conjunction with p65-based domains. Polypeptides which can function to activate transcription in eukaryotic cells are well known in the art. In particular, transcription activation domains have been described for many transcription factors and have been shown to retain their activation function when the transcription activation domain, or a suitable fragment thereof, is present within a fusion protein.

Activation domains can comprise naturally occurring or non-naturally occurring peptide sequences, so long as, either alone or in combination with other activation domains, they are capable of activating transcription. Any particular activation domain is preferably at least 6 amino acids in length. Naturally occurring activation domain subunits or motifs include portions of transcription factors, such as a thirty amino acid fragment of the C-terminus of VP16 (amino acids 461-490), referred to herein as "Vc". Other activation domain subunits are derivatives of naturally occurring peptides. For example, the replacement of one amino acid of a naturally occurring activation unit by another may further increase activation. An example of such an activation unit is a derivative of an eight amino acid peptide of VP16, the derivative having the amino acid sequence DFDLDMLG. [Seq. ID No. 3]

Yet other activation units are entirely synthetic. It is known, for example, that certain random alignments of acidic amino acids are capable of activating transcription.

It is well known in the art that certain transcription factors are active only in specific cell types. By using tissue specific activation domains, it is possible to design a transcription factor having a certain tissue specificity.

One source of polypeptide motifs for use in conjunction with p65-based activation domains is the herpes simplex virus virion protein 16 (referred to herein as VP16, the amino acid sequence of which is disclosed in Triezenberg, S.J. et al. (1988) Genes Dev. 2:718-729). In one embodiment, an activation domain corresponding to about 127 of the C-terminal amino acids of VP16 is used. For example, a polypeptide having amino acid residues 208-335 can be used as an auxiliary activation domain. In another embodiment, at least one copy of about 11 amino acids from the C-terminal region of VP16 which retain transcription activation ability is used as an additional activation domain. Preferably, an oligomer of this region (i.e., about 22 amino acids) is used. Suitable C-terminal peptide portions of VP16 are

described in Seipel, K. et al. (EMBO J. (1992) 13:4961-4968). VP16-derived transcription activation domains have been used successfully in many of the different regulated expression systems referred to herein.

Another example of an acidic activation domain is provided in residues 753-881 of GAL4. A preferred activation domain to be used in conjunction with a p65 domain is a domain from a human heat shock factor transcriptional activator (HSF domain), in particular, residues 406-530. Heat shock factor activation domains are more fully described in USSN 09/262,600, filed 3/4/99, the full contents of which are incorporated herein by reference.

Other illustrative activation domains and motifs of human origin include the activation domain of human CTF, the 18 amino acid (NFLQLPQQTQGALLTSQP) [Seq. ID No. 4] glutamine rich region of Oct-2, the N-terminal 72 amino acids of p53, the SYGQQS [Seq. ID No. 5] repeat in Ewing sarcoma gene and an 11 amino acid (535-545) acidic rich region of Rel A protein. Various additional activation domains, motifs and chimeric transcription factors are provided in the examples which follow. See also USSN 08/920,610 (ARIAD 363-A), the contents of which are incorporated herein by reference, especially for additional information concerning sources of activation domains and motifs that may be used in combination with p65 domains in the chimeric transcription domains of this invention.

2. Ligand binding domains

The chimeric transcription factors contain at least one p65 domain and one ligand binding domain, but function, in the various embodiments, through different molecular mechanisms.

A. Dimerization-based systems

In certain embodiments, the ligand binding domain permits ligand-mediated cross-linking of the chimeric transcription factor with a second fusion protein (which contains at least one ligand binding domain and DNA binding domain). In these cases, the ligand is at least divalent and functions as a dimerizing agent by binding to the two fusion proteins and forming a cross-linked heterodimeric complex which activates target target gene expression. See e.g. WO 94/18317, WO 96/20951, WO 96/06097, WO 97/31898, WO 96/41865, and PCT US98/17723, the contents of which are incorporated herein by reference.

In other embodiments, the ligand binding event is thought to result in an allosteric change in the chimeric transcription factor leading to binding of the fusion protein to a target DNA sequence [see e.g. US 5,654,168 and 5,650,298 (tet systems), and WO 93/23431 and WO 98/18925 (RU486-based systems)] or to another protein [see e.g. WO 96/37609 and

pCGNNZFHD1-FKBPx1 and pCGNNZFHD1-FKBPx3, were prepared containing one or three tandem repeats of human FKBP12 ligated as an XbaI-BamHI fragment between the Spe1 and BamHI sites of pCGNNZFHD1. A sample of pCGNNZFHD1-FKBPx3 has been deposited with the American Type Culture Collection under ATCC Accession No. 97399.)

Primers:

5'Xba/Zif	5'ATGCTCTAGAGAACGCCCATATGCTTGCCCT	[Seq. ID No. 6]
3'Zif+G	5'ATGCGCGGCCGCCGCCTGTGTGGGTGCGGATGTG	[Seq. ID No. 7]
5'Not OctHD	5'ATGCGCGGCCGCAGGAGGAAGAAACGCACCAGC	[Seq. ID No. 8]
Spe/Bam 3'Oct	5'GCATGGATCCGATTCAACTAGTGTTGATTCTTTTTTCTTTCTGGCGGCG	[Seq. ID No. 9]

To generate C-terminal fusions of FRB domain(s) with the chimeric DNA binding protein ZFHD1, the XbaI-BamHI fragments encoding 1, 2, 3 and 4 copies of FRB were recovered from the GAL4 fusion vectors and ligated into Spe-BamHI digested pCGNN-ZFHD1 to yield pCGNN-ZFHD1-1FRB, pCGNN-ZFHD1-2FRB etc. Constructs were verified by restriction analysis and/or DNA sequencing.

To examine the effect of introducing additional 'linker' polypeptide between ZFHD1 and a C-terminal FRB domain, FRAP fragments encoding extra sequence N-terminal to FRB were cloned as ZFHD1 fusions. XbaI-BamHI fragments encoding $FRAP_a$, $FRAP_b$, $FRAP_c$, $FRAP_d$ and $FRAP_e$ were excised from the vectors pCGNN-GAL4-$FRAP_a$, pCGNN-GAL4-$FRAP_b$ etc and ligated into SpeI-BamHI digested pCGNN-ZFHD1 to yield the vectors pCGNN-ZFHD1-$FRAP_a$, pCGNN-ZFHD1-$FRAP_b$, etc. Vectors encoding fusions of ZFHD1 to 2, 3 and 4 C-terminal copies of $FRAP_e$ were also constructed by isolating XbaI-BamHI fragments encoding $2FRAP_e$, $3FRAP_e$ and $4FRAP_e$ from pCGNN-GAL4-$2FRAP_e$, pCGNN-GAL4-$3FRAP_e$ and pCGNN-GAL4-$4FRAP_e$ and ligating them into SpeI-BamHI digested pCGNN-ZFHD1 to yield the vectors pCGNN-ZFHD1-$2FRAP_e$, pCGNN-ZFHD1-$3FRAP_e$ and pCGNN-ZFHD1-$4FRAP_e$. All constructs were verified by restriction analysis.

Vectors were also constructed that encode N-terminal fusions of FRB domain(s) with ZFHD1. XbaI-BamHI fragments encoding 1, 2, 3 and 4 copies of $FRAP_e$ were isolated from pCGNN-GAL4-$1FRAP_e$, pCGNN-GAL4-$2FRAP_e$ etc and ligated into XbaI-BamHI digested pCGNN to yield the plasmids pCGNN-$1FRAP_e$, pCGNN-$2FRAP_e$ etc. These vectors were then digested with SpeI and BamHI, and an XbaI-BamHI fragment encoding ZFHD1 (isolated from pCGNN-ZFHD1) ligated in to yield the constructs pCGNN-$1FRAP_e$-ZFHD1, pCGNN-$2FRAP_e$-ZFHD1 etc, which were verified by restriction analysis.

(f) Constructs encoding FRAP-p65 activation domain fusions

To generate fusions of FRB domain(s) with the activation domain of the human NF-kb p65 subunit (hereafter designated p65), two fragments were amplified by PCR from the plasmid pCG-p65. Primers **9** (p65/ 5' Xba) and **11** (p65 3' Spe/Bam) amplify the coding sequence for amino acids 450-550, and primers **10** (p65/361 Xba) and **11** amplify the coding sequence for amino acids 361-550, both flanked by 5' XbaI and 3' SpeI/BamHI sites. PCR products were digested with XbaI and BamHI and cloned into XbaI-BamHI digested pCGNN to yield pCGNN-p65(450-550) and pCGNN-p65(361-550). The constructs were verified by restriction analysis and DNA sequencing.

The 100 amino acid P65 transcription activation sequence is encoded by the following linear sequence:

CTGGGGGCCTTGCTTGGCAACAGCACAGACCCAGCTGTGTTCACAGACCTGGCATCCGTCGACAA
CTCCGAGTTTCAGCAGCTGCTGAACCAGGGCATACCTGTGGCCCCCCACACAACTGAGCCCATGC
TGATGGAGTACCCTGAGGCTATAACTCGCCTAGTGACAGGGGCCCAGAGGCCCCCCGACCCAGCT
CCTGCTCCACTGGGGGCCCCGGGGCTCCCCAATGGCCTCCTTTCAGGAGATGAAGACTTCTCCTC
CATTGCGGACATGGACTTCTCAGCCCTGCTGAGTCAGATCAGCTCC [Seq. ID No. 10]

The more extended p65 transcription activation domain (351-550) is encoded by the following linear sequence:

GATGAGTTTCCCACCATGGTGTTTCCTTCTGGGCAGATCAGCCAGGCCTCGGCCTTGGCCCCGGCC
CCTCCCCAAGTCCTGCCCCAGGCTCCAGCCCCTGCCCCTGCTCCAGCCATGGTATCAGCTCTGGC
CCAGGCCCCAGCCCCTGTCCCAGTCCTAGCCCCAGGCCCTCCTCAGGCTGTGGCCCCACCTGCCC
CCAAGCCCACCCAGGCTGGGGAAGGAACGCTGTCAGAGGCCCTGCTGCAGCTGCAGTTTGATGAT
GAAGACCTGGGGGCCTTGCTTGGCAACAGCACAGACCCAGCTGTGTTCACAGACCTGGCATCCGT
CGACAACTCCGAGTTTCAGCAGCTGCTGAACCAGGGCATACCTGTGGCCCCCCACACAACTGAGC
CCATGCTGATGGAGTACCCTGAGGCTATAACTCGCCTAGTGACAGCCCAGAGGCCCCCCGACCCA
GCTCCTGCTCCACTGGGGGCCCCGGGGCTCCCCAATGGCCTCCTTTCAGGAGATGAAGACTTCTC
CTCCATTGCGGACATGGACTTCTCAGCCCTGCTGAGTCAGATCAGCTCCTAA [Seq. ID No.11]

To generate N-terminal fusions of FRB domain(s) with portions of the p65 activation domain, plasmids pCGNN-1FRB, pCGNN-2FRB etc were digested with SpeI and BamHI. An XbaI-BamHI fragment encoding p65 (450-550) was isolated from pCGNN-p65(450-550) and ligated into the SpeI-BamHI digested vectors to yield the plasmids pCGNN-1FRB-p65(450-

products are purified, digested with XbaI and SpeI, ligated into XbaI-SpeI digested pCGNN, and verified by restriction analysis and DNA sequencing.

(h) Primer sequences

1	5' GCATGTCTAGAGAGATGTGGCATGAAGGCCTGGAAG	[Seq. ID No. 12]
2	5' GCATCACTAGTCTTTGAGATTCGTCGGAACACATG	[Seq. ID No. 13]
3	5' GCACATTCTAGAATTGATACGCCCAGACCCTTG	[Seq. ID No. 14]
4	5' CGATCAACTAGTAAGTGTCAATTTCCGGGGCCT	[Seq. ID No. 15]
5	5' GCACTATCTAGACTGAAGAACATGTGTGAGCACAGC	[Seq. ID No. 16]
6	5' GCACTATCTAGAGTGAGCGAGGAGCTGATCCGAGTG	[Seq. ID No. 17]
7	5' CGATCAACTAGTGGAAACATATTGCAGCTCTAAGGA	[Seq. ID No. 18]
8	5' CGATCAACTAGTTGGCACAGCCAATTCAAGGTCCCG	[Seq. ID No. 19]
9	5' ATGCTCTAGACTGGGGGCCTTGCTTGGCAAC	[Seq. ID No. 20]
10	5' ATGCTCTAGAGATGAGTTTCCCACCATGGTG	[Seq. ID No. 21]
11	5' GCATGGATCCGCTCAACTAGTGGAGCTGATCTGACTCAG	[Seq. ID No. 22]
12	5' ATGCTCTAGACTTGGAACCGGACCTGCCGCC	[Seq. ID No. 23]
13	5' GCATCACTAGTCCAGAAAGGGCACCAGCCAATAT	[Seq. ID No. 24]

Restriction sites are underlined (XbaI = TCTAGA, SpeI = ACGAGT, BamHI = GGATCC).

(i) DNA sequence of representative final construct: pCGNN-ZFHD1-1FRB

12CA5

epitope

M A S S Y P Y D V P D

5' gtagaagcgcgt ATG GCT TCT AGC TAT CCT TAT GAC GTG CCT GAC

SV40 T NLS

Y A S L G G P S S P K K K R K

TAT GCC AGC CTG GGA GGA CCT TCT AGT CCT AAG AAG AAG AGA AAG

(X/S)

ZFHD1(5')

V S R E R P Y A C P V E S C D...

GTG TCT AGA GAA CGC CCA TAT GCT TGC CCT GTC GAG TCC TGC GA...

XbaI

ZFHD1(3') FRB(5')

...R I N T R E M W H E G L E E...

...AGA ATC AAC ACT AGA GAG ATG TGG CAT GAA GGC CTG GAA GA...

(S/X)

FRB(3')

R I S K T S Y *

CGA ATC TCA AAG ACT AGT TAT TAG ggatcctgag

SpeI BamHI

[Seq. ID No. 25]

[Seq. ID No. 26]

Non-coding nucleotides are indicated in lower case

(S/X) and (X/S) indicate the result of a ligation event between the compatible products of digestion with XbaI and SpeI, to produce a sequence that is cleavable by neither enzyme

* indicates a stop codon

(j) Bicistronic constructs

The internal ribosome entry sequence (IRES) from the encephalomyocarditis virus was amplified by PCR from pWZL-Bleo. The resulting fragment, which was cloned into pBS-SK+ (Stratagene), contains an XbaI site and a stop codon upstream of the IRES sequence and downstream of it, an NcoI site encompassing the ATG followed by SpeI and BamHI sites. To facilitate cloning, the sequence around the initiating ATG of pCGNN-ZFHD1-3FKBP was mutated to an NcoI site and the XbaI site was mutated to a NheI site using the oligonucleotides

5'-GAATTCCTAGAAGCGACCATGGCTTCTAGC-3' [Seq. ID No. 27]

and

5'-GAAGAGAAAGGTGGCTAGCGAACGCCCATAT-3' [Seq. ID No. 28]

respectively. An NcoI-BamHI fragment containing ZFHD1-3FKBP was then cloned downstream of pBS-IRES to create pBS-IRES-ZFHD1-3FKBP. The XbaI-BamHI fragment from this plasmid was next cloned into SpeI/BamHI-cut pCGNN-1FRB-p65(361-550) to create pCGNN-1FRB-p65(361-550)-IRES-ZFHD1-3FKBP.

PCR primers for the HSF trimerization domain:

5'primer: atgctctagaagtgtgtccaccctgaagagtgaagac [Seq. ID No. 29]

3' primer: atgctgatcaagatctttattaactagtgccactgtcgttcagcatcaggggat [Seq. ID No. 30]

Template: pBS108 vector containing human HSF1 full length cDNA.

The PCR fragments containing the trimerization domain of HSF1 (amino acids 120-217) will be digested with Xba1/Bcl1 and cloned into Spe1/BamH1 digested PCGNN-FRB vectors. The resulting PCGNN-FRB-T vectors will be digested with Spe1-Bgl II and ligated with fragments containing p65 activation domain (amino acids 361-550) or constructs containing both the p65 activation domain and the HSF activation domain (amino acids 431-529).

2. Retroviral vectors for the expression of chimeric proteins

Retroviral vectors used to express transcription factor fusion proteins from stably integrated, low copy genes were derived from pSRaMSVtkNeo (Muller et al., MCB 11:1785-92, 1991) and pSRaMSV(XbaI) (Sawyers et al., J. Exp. Med. 181:307-313, 1995). Unique BamHI sites in both vectors were removed by digesting with BamHI, filling in with Klenow and religating to produce pSMTN2 and pSMTX2, respectively. pSMTN2 expresses the Neo

gene from an internal thymidine kinase promoter. A Zeocin gene (Invitrogen) will be cloned as a NheI fragment into a unique XbaI site downstream of an internal thymidine kinase promoter in pSMTX2 to yield pSNTZ. This Zeocin fragment was generated by mutagenizing pZeo/SV (Invitrogen) using the following primers to introduce NheI sites flanking the zeocin coding sequence.

Primer 1 5'-GCCATGGTGGCTAGCCTATAGTGAG [Seq. ID No. 31]

Primer2 5'-GGCGGTGTTGGCTAGCGTCGGTCAG [Seq. ID No. 32]

pSMTN2 contains unique EcoRI and HindIII sites downstream of the LTR. To facilitate cloning of transcription factor fusion proteins synthesized as XbaI-BamHI fragments the following sequence was inserted between the EcoRI and HindIII sites to create pSMTN3:

12CA5 epitope

M A S S Y P Y D V P D

5' gaattccagaagcgcgt ATG GCT TCT AGC TAT CCT TAT GAC GTG CCT GAC

EcoRI

SV40 T NLS

Y A S L G G P S S P K K K R K

TAT GCC AGC CTG GGA GGA CCT TCT AGT CCT AAG AAG AAG AGA AAG

[Seq. ID No. 33]

V

GTG TCT AGA TAT CGA GGA TCC CAA GCT T [Seq. ID No. 34]

XbaI BamHI HindIII

The equivalent fragment is inserted into a unique EcoRI site of pSMTZ to create pSMTZ3 with the only difference being that the 3' HindIII site is replaced by an EcoRI site. pSMTN3 and pSMTZ3 permit chimeric transcription factors to be cloned downstream of the 5' viral LTR as XbaI-BamHI fragments and allow selection for stable integrants by virtue of their ability to confer resistance to the antibiotics G418 or Zeocin respectively.

To generate the retroviral vector SMTN-ZFHD1-3FKBP, pCGNN-ZFHD1-3FKBP was first mutated to add an EcoRI site upstream of the first amino acid of the fusion protein. An EcoRI-BamHI(blunted) fragment was then cloned into EcoRI-HindIII(blunted) pSRaMSVtkNeo (ref. 51) so that ZFHD1-3FKBP was expressed from the retroviral LTR.

medium containing the indicated amounts of rapamycin was added to each well. After 24 hours, medium was removed and assayed for SEAP activity as described (Spencer et al, Science 262:1019-24, 1993) using a Luminescence Spectrometer (Perkin Elmer) at 350 nm excitation and 450 nm emission. Background SEAP activity, measured from mock-transfected cells, was subtracted from each value.

To prepare transiently transfected HT1080 cells for injection into mice (See below), cells in 100 mm dishes (2 x 10^6 cells/dish) were transfected by calcium phosphate precipitation for 16 hours (Gatz, C., Kaiser, A. & Wendenburg, R. , 1991,*Mol. Gen. Genet.* **227**, 229-237) with the following DNAs: 10 mg of ZHWTx12-CMV-hGH, 1 mg pCGNN-ZFHD1-3FKBP, 2 mg pCGNN-1FRB-p65(361-550) and 7 mg pUC118. Transfected cells were rinsed 2 times with phosphate buffered saline (PBS) and given fresh medium for 5 hours. To harvest for injection, cells were removed from the dish in Hepes Buffered Saline Solution containing 10 mM EDTA, washed with PBS/0.1% BSA/0.1% glucose and resuspended in the same at a concentration of 2 x 10^7 cells/ml.

Plasmids:

Construction of the transcription factor fusion plasmids is described above.

pZHWTx12-CMV-SEAP

This reporter gene, containing 12 tandem copies of a ZFHD1 binding site (Pomerantz et al., 1995) and a basal promoter from the immediate early gene of human cytomegalovirus (Boshart et. al., 1985) driving expression of a gene encoding secreted alkaline phosphatase (SEAP), was prepared by replacing the NheI-HindIII fragment of pSEAP Promoter (Clontech) with the following NheI-XbaI fragment containing 12 ZFHD binding sites:

GCTAGCTAATGATGGGCGCTCGAGTAATGATGGGCGGTCGACTAATGATGGGCGCTCGAGTAATGATGGGCGTC
TAGCTAATGATGGGCGCTCGAGTAATGATGGGCGGTCGACTAATGATGGGCGCTCGAGTAATGATGGGCGTCTA
GCTAATGATGGGCGCTCGAGTAATGATGGGCGGTCGACTAATGATGGGCGCTCGAGTAATGATGGGCGTCTAGA
[Seq. ID No. 35]

(the ZFHD1 binding sites are underlined),

and the following XbaI-HindIII fragment containing a minimal CMV promoter (-54 to +45):

TCTAGAACGCGAATTCCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAG
ATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGCTT [Seq. ID No. 36]

(the CMV minimal promoter is underlined).

pZHWTx12-CMV-hGH

Activation of this reporter gene leads to the production of hGH. It was constructed by replacing the HindIII-BamHI (blunted) fragment of pZHWTx12-CMV-SEAP (containing the SEAP coding sequence) with a HindIII (blunted) -EcoRI fragment from p0GH (containing an hGH genomic clone; Selden et al., MCB 6:3171-3179, 1986; the BamHI and EcoRI sites were blunted together).

pZHWTx12-IL2-SEAP

This reporter gene is identical to pZHWTx12-CMV-SEAP except the XbaI-HindIII fragment containing the minimal CMV promoter was replaced with the following XbaI-HindIII fragment containing a minimal IL2 gene promoter (-72 to +45 with respect to the start site; Siebenlist et al., MCB 6:3042-3049, 1986):

TCTAGAACGCGAATTCAACATTTTGACACCCCCATAATATTTTTCCAGAATTAACAGTATAAATTGCATCTCTT
GTTCAAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCCTGCCACAAGCTT

[Seq. ID No. 37]

(the IL2 minimal promoter is underlined).

pLH

To facilitate the stable integration of a single, or few, copies of reporter gene the following retroviral vector was constructed. pLH (LTR-*hph*), which contains the hygromycin B resistance gene driven by the Moloney murine leukemia virus LTR and a unique internal ClaI site, was constructed as follows: The hph gene was cloned as a HindIII-ClaI fragment from pBabe Hygro (Morganstern and Land, NAR 18:3587-96, 1990) into BamHI-ClaI cut pBabe Bleo (resulting in the loss of the bleo gene; the BamHI and HindIII sites were blunted together).

pLH-ZHWTx12-IL2-SEAP

To clone a copy of the reporter gene containing 12 tandem copies of the ZFHD1 binding site and a basal promoter from the IL2 gene driving expression of the SEAP gene into the pLH retroviral vector, the MluI-ClaI fragment from pZHWTx12-IL2-SEAP (with ClaI linkers added) was cloned into the ClaI site of pLH. It was oriented such that the directions of transcription from the viral LTR and the internal ZFHD-IL2 promoters were the same.

pLH-G5-IL2-SEAP

To construct a retroviral vector containing 5 Gal4 sites embedded in a minimal IL2 promoter driving expression of the SEAP gene, a ClaI-BstBI fragment consisting of the following was inserted into the ClaI site of pLH such that the directions of transcription from the viral LTR

and the internal Gal4-IL2 promoters were the same: A ClaI-HindIII fragment containing 5 Gal4 sites (underlined) and regions -324 to -294 (bold) and -72 to +45 of the IL2 gene (italics)

5'ATCGATG**TTTTCTGAGTTACTTTTGTATCCCCACCCCCC**CTCGAGCTTGCATGCCTGCAGGTCGGAG
TACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGG
AGTACTGTCCTCCGAGCGCAGACTCTAGAGGATCCGAGAACATTTTGACACCCCCATAATATTTTTCCAGAATT
AACAGTATAAATTGCATCTCTTGTTCAAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAAC
TCCTGCCACAAGCTT, [Seq. ID No. 38]

and a HindIII-BstBI fragment containing the SEAP gene coding sequence (Berger et al., Gene 66:1-10, 1988) mutagenized to add the following sequence (containing a BstB1 site) immediately after the stop codon:

5'-CCCGTGGTCCCGCGTTGCTTCGAT [Seq. ID No. 39]

5. Rapamycin-dependent transcriptional activation in stably transfected cells

We conducted the following experiments to confirm that this system exhibits similar properties in stably transfected cells. We generated stable cell lines by sequential transfection of a SEAP target gene and expression vectors for ZFHD1-3FKBP and 1FRB-p65, respectively. A pool of several dozen stable clones resulting from the final transfection exhibited rapamycin-dependent SEAP production. From this pool, we characterized several individual clones, many of which produced high levels of SEAP in response to rapamycin. One such clone produced SEAP at levels approximately forty times higher than the pool and significantly higher than transiently transfected cells. In an attempt to rigorously quantitate background SEAP production and induction ratio in this clone, we performed a second set of assays in which the length of the SEAP assay was increased by a factor of approximately 50 to detect any SEAP activity in untreated cells. Under these conditions, mock transfected cells produced 47 arbitrary fluorescence units, while the transfected clone produced 54 units in the absence of rapamycin and over 90,000 units at 100 nM rapamycin. Thus, in this stable cell line, background gene expression was negligible and the induction ratio (7 units to 90,000 units) was greater than four orders of magnitude.

To simplify the task of stable transfection, we used a bicistronic expression vector that directs the production of both ZFHD1-3FKBP and 1FRB-p65 through the use of an internal ribosome entry sequence (IRES). This expression plasmid was cotransfected, together with a zeocin-resistance marker plasmid, into a cell line carrying a retrovirally-transduced SEAP reporter gene, and a pool of approximately fifty drug-resistant clones was selected and

pCGNN (Attar and Gilman, 1992) to make pCGNNZFHD1 in which the cDNA insert is under the transcriptional control of human CMV promoter and enhancer sequences and is linked to the nuclear localization sequence from SV40 T antigen. The plasmid pCGNN also contains a gene for ampicillin resistance which can serve as a selectable marker.

pCGNNZFHD1-p65

An expression vector for directing the expression in mammalian cells of a chimeric transcription factor containing the composite DNA-binding domain, ZFHD1, and a transcription activation domain from p65 (human) was prepared as follows. The sequence encoding the C-terminal region of p65 containing the activation domain (amino acid residues 450-550) was amplified from pCGN-p65 using primers p65 5' Xba and p65 3' Spe/Bam. The PCR fragment was digested with Xba1 and BamH1 and ligated between the the Spe1 and BamH1 sites of pCGNN ZFHD1 to form pCGNN ZFHD-p65AD.

The P65 transcription activation sequence contains the following linear sequence:

CTGGGGGCCTTGCTTGGCAACAGCACAGACCCAGCTGTGTTCACAGACCTGGCATCCGTCGACAACTCCGAGTT
TCAGCAGCTGCTGAACCAGGGCATACCTGTGGCCCCCCACACAACTGAGCCCATGCTGATGGAGTACCCTGAGG
CTATAACTCGCCTAGTGACAGGGGCCCAGAGGCCCCCCGACCCAGCTCCTGCTCCACTGGGGGCCCCGGGGCTC
CCCAATGGCCTCCTTTCAGGAGATGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCAGCCCTGCTGAGTCA
GATCAGCTCC [Seq. ID No. 40]

pCGNNZFHD1-FKBPx3

An expression vector for directing the expression of ZFHD1 linked to three tandem repeats of human FKBP was prepared as follows.Three tandem repeats of human FKBP were isolated as an XbaI-BamHI fragment from pCGNNF3 and ligated between the Spe1 and BamHI sites of pCGNNZFHD1 to make pCGNNZFHD1-FKBPx3 (ATCC Accession No. 97399).

pZHWTx8SVSEAP

A reporter gene construct containing eight tandem copies of a ZFHD1 binding site (Pomerantz *et al.*, 1995) and a gene encoding secreted alkaline phosphatase (SEAP) was prepared by ligating the tandem ZFHD1 binding sites between the Nhe1 and BglII sites of pSEAP-Promoter Vector (Clontech) to form pZHWTx8SVSEAP. The ZHWTx8SEAP reporter contains two copies of the following sequence in tandem:

CTAGCTAATGATGGGCGCTCGAGTAATGATGGGCGGTCGACTAATGATGGGCGCTCGAGTAATGATGGGCGT

[Seq. ID No. 41]

The ZFHD1 binding sites are underlined.

pCGNN F1 and F2

One or two copies of FKBP12 were amplified from pNF3VE using primers FKBP 5' Xba and FKBP 3' Spe/ Bam. The PCR fragments were digested with Xba1 and BamH1 and ligated between the Xba1 and BamH1 sites of pCGNN vector to make pCGNN F1 or pPCGNN F2. pCGNNZFHD1-FKBPx3 can serve as an alternate source of the FKBP cDNA.

pCGNN F3

A fragment containing two tandem copies of FKBP was excised from pCGNN F2 by digesting with Xba1 and BamH1. This fragment was ligated between the Spe1 and BamH1 sites of pCGNN F1.

pCGNN F3VP16

The C-terminal region of the Herpes Simplex Virus protein, VP16 (AA 418-490) containing the activation domain was amplified from pCG-Gal4-VP16 using primers VP16 5' Xba and VP16 3' Spe/Bam. The PCR fragment was digested with Xba1 and BamH1 and ligated between the Spe1 and BamH1 sites of pCGNN F3 plasmid.

pCGNN F3p65

The Xba1 and BamH1 fragment of p65 containing the activation domain was prepared as described above. This fragment was ligated between the Spe1 and BamH1 sites of pCGNN F3.

Primers

5'Xba/Zif	5'ATGCTCTAGAGAACGCCCATATGCTTGCCCT	[Seq. ID No. 42]
3'Zif+G	5'ATGCGCGGCCGCCGCCTGTGTGGGTGCGGATGTG	[Seq. ID No. 43]
5'Not OctHD	5'ATGCGCGGCCGCAGGAGGAAGAAACGCACCAGC	[Seq. ID No. 44]
Spe/Bam 3'Oct	5'GCATGGATCCGATTCAACTAGTGTTGATTCTTTTTTCTTTCTGGCGGCG	[Seq. ID No. 45]
FKBP 5'Xba	5'TCAGTCTAGAGGAGTGCAGGTGGAAACCAT	[Seq. ID No. 46]
FKBP 3' Spe/Bam	5'TCAGGGATCCTCAATAACTAGTTTCCAGTTTTAGAAGCTC	[Seq. ID No. 47]

VP16 5' Xba	5'ACTGTCTAGAGTCAGCCTGGGGGACGAG	[Seq. ID No. 48]
VP16 3' Spe/Bam	5'GCATGGATCCGATTCAACTAGTCCCACCGTACTCGTCAATTCC	[Seq. ID No. 49]
P65 5' Xba	5'ATGCTCTAGACTGGGGGCCTTGCTTGGCAAC	[Seq. ID No. 50]
p65 3' Spe/Bam	5'GCATGGATCCGCTCAACTAGTGGAGCTGATCTGACTCAG	[Seq. ID No. 51]

References

1. Attar, R.M., and M.Z. Gilman 1992. *Mol. Cell. Biol.* 12:2432-2443
2. Ausubel, F.M. *et al.*, Eds., 1994. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley, NY)
3. Pomerantz, J.L., *et al.* 1995. *Science.* 267:93-96.
4. Spencer, D.M., *et al.* 1993. *Science.* 262:1019-1024.

II. Evaluation of representative illustrative chimeric transcription factors

Constructs

Constructs encoding the following GAL-4-based chimeric transcription factors, among others, were prepared and tested in human cell lines containing stably integrated SEAP reporter constructs containing GAL4 or ZFHD1 recognition sequences, as appropriate:

chimeric factor	data shown in Figure
G-K	Fig. 2
G-KK	
G-KKK	
G-KKKK	
G-KKKKK	
G-KKKKKK	
G-(V8x2)	Fig. 3
G-(V8x2)$_2$	
G-(V8x2)$_3$	
G-(V8x2)$_4$	
G-(V8x2)$_5$	
G-(V8x2)$_6$	

(continued —>)

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chimeric factor	data shown in Figure
G-D	Fig. 4
G-DD	
G-DDD	
G-DDDD	
G-DDDDD	
G-DDDDDD	
Z-VP16	Fig. 5
Z-k	
Z-kkk	
Z-K	
Z-KKK	
G-KKK-(V8x2)4	Fig. 6
G-KKK-DDDDD	
G-(V8x2)4-DDDDD	
G-KKK-(V8x2)4-DDDDD	
G-K	Fig. 7
G-KKK	
G-HSF-HSF	
G-HSF-HSF-HSF-HSF	
G-K-HSF-HSF-HSF-HSF	
G-KKK-HSF-HSF-HSF-HSF	

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abbreviations: G = GAL4 residues 1-94

K = p65(361-550) = "N361" in Fig. 6

k = p65(450-550) = "N450" in Fig. 6

V8x2 = tandem repeat of VP16 V8 sequence with an intervening SerArg resulting from ligation; (V8x2)4="8V8" in Fig 6

D = VP16 C terminal SRDFDLDMLG [Seq. ID No. 52] containing an initial SerArg resulting from ligation = "Vc" in Fig 6

Z = ZFHD1 ("ZH" in Fig 5)

HSF = 14 mer (see table below)

Plasmid constructions: PCG-Gal4 vector containing Gal4 DNA binding domain coding sequences between amino acids 1-94 was digested with Xba1 and BamH1. The p65 activation domain sequences between amino acids 361-550 was generated by PCR using the following oligonuleotides:

5'-atgctctagagatgagtttcccaccatggtg-3' [Seq. ID No. 53]

and

5'-gcatggatccgctcaactagtggagctgatctgactcag-3'. [Seq. ID No. 54]

This fragment was digested with Xba1 and BamH1 and cloned into PCG-Gal4 vector to make PCG-Gal4-p65 (361-550), here after will be referred as PCG-GK. To make PCG-GK2 plasmid, the p65 activation domain containing PCR fragment described above was digested with Xba1 and BamH1 and cloned into Spe1 and BamH1 digested PCG-GK vector. PCG-GK3, 4, 5,6 were all generated following the same approach.

Plasmid PCG-Gal 4 plasmids containing reiterated copies of V8 domain were generated by the following method. The oligonucleotides 5'-ctagagacttcgacttggacatgct-3' [Seq. ID No. 55]; 5'agtcccccagcatgtccaagtcgaagtct-3' [Seq. ID No. 56]; 5'-gggggacttcgacttggacatgctgactagttgag-3' [Seq. ID No. 57] and 5'-gatcctcaactagtcagcatgtccaagtcga-3' [Seq. ID No. 58] were phosphorylated and the first and last pair of oligos were annealed seperately. Together these oligonucleotides make two tandem V8 coding sequences. These annealed oligos were then ligated into Xba1 and BamH1 digested PCG-Gal4 vector. The resulting vector, PCG-GV2 containing two copies of V8 sequences was digested with Spe1 and BamH1. V8x2 oligos made as described above was cloned into this vector to make PCG-GV4. Same approach was taken to generate PCG-GV6, 8, 10 and 12 plasmids.

PCG-Gal4 plasmids containing reiterated copies of VP16 C-terminus, hereafter refered as D activation domain were constructed as follows. The VP16 C-terminus region was PCR amplified using the following primers:
5'-atgctctagagacggggattccccggggccg-3' [Seq. ID No. 59] and
5'gcatggatcctcattaactagtcccaccgtactcgtcaattcc-3' [Seq. ID No. 60]. The PCR fragments were digested with Xba1 and BamH1 and cloned into PCG-Gal4 vector previously digested with Xba1 and BamH1. The resulting plasmid was designated as PCG-GD. To make PCG-GD2, PCG-GD was digested with Spe1 and BamH1 and ligated with Xba1 and BamH1 digested D fragment described above. PCG-GD3,4,5 and 6 were constructed using the same approach.

Plasmids PCG-GK3V8 and PCG-GK3D5 were made by digesting PCG-GV8 and PCG-D5 plasmids with Xba1 and BamH1 and cloning the fragments containing V8 and D5 sequences respectively into PCG-GK3 digested with Spe1 and BamH1. Similarly, Xba1/BamH1 fragment from PCG-GD5 containing D5 sequences was cloned into Spe1/BamH1 digested PCG-GV8 plasmid to construct PCG-V8D5 plasmid. The V8D5 fragment was excised from this plasmid by digesting it with Xba1 and BamH1 and the fragment was cloned into Spe1/BamH1 digested PCG-K3 to make PCG-K3V8D5 plasmid.

PCGNN-ZFHD-p65(450-550) and PCGNN-ZFHD-p65(361-550) are described above. PCGNN-p65(450-550)x3 and PCGNN-ZFHD-p65(361-550) were made as follows: PCG-Gal4-p65(450-550)x3 and PCG-Gal4-p65(361-550) were digested with Xba1 and BamH1 and the p65(450-550)x3 and p65(361-550) were excised. These fragments were cloned into Spe1/BamH1 digested PCGNN-ZFHD to generate PCGNN-ZFHD-p65(450-550) and PCGNN-ZFHD-p56(361-550).

PCG-Gal4-HSFX2 containing two copies of HSF14 activation domain was made by phosphorylating and ligating the following oligonucleotides to Xba1 and BamH1 digested PCG-Gal4 plasmid:

5'-ctagagacaccagtgccctgctggacctgttcagcccctcg-3'; [Seq. ID No. 61]

5'-ggtcaccgaggggctgaacaggtccagcagggcactggtgtct-3'; [Seq. ID No. 62]

5'-gtgaccgtgcccgacatgagcctgcctgaccttgacagcag-3' and [Seq. ID No. 63]

5'-gtgaccgtgcccgacatgagcctgcctgaccttgacagcag-3'. [Seq. ID No. 64]

Two additional copies of HSF activation domain were added to Spe1/BamH1 digested PCG-Gal4-HSFX2 plasmid by the same method to generate PCG-Gal4-HSFX4 plasmid. A fragment containing four copies of HSF14 activation domain was excised from PCG-Gal4-HSFX4 by Xba1 and BamH1 digestion. The resulting fragment was cloned into Spe1 and BamH1 digested PCG-Gal4KX1 and PCG-Gal4KX3 to to make PCG-Gal4-K+HSFX4 or PCG-Gal4-K3+HSFX4 plasmids.

reporter cell lines

Human 1080 cells were engineered by the stable introduction of a secreted alkaline phosphatatse (SEAP) target gene construct. The target gene construct contained a gene encoding SEAP operably linked to a transcription control sequence containing five copies of a DNA recognitions sequence for GAL4 and a minimal IL-2 promoter. The resultant cells may be

Representative results:

chimeric transcription factor	number of activation domains	transcription activation (IL2 promoter)
GAL4-p65(361-550)	1 to 6	++++
GAL4-p65(450-550)	1 to 6	+++
GAL4-p65(361-450)	1 to 6	--
GAL4-K13 (SRDFADMDFDALL [Seq. ID No. 65], derived from p65)	1 to 6	+++
GAL4-Oct2 Q domain (aa95-160)	1 to 6	--
GAL4-Oct2 P domain (aa438-479)	1 to 6	--
GAL4-HSF (aa 409-444)	1 to 4	+++
GAL4-HSF14 (DLDSSLASIQELLS) [Seq. ID No. 66]	1 to 4	++
GAL4-EWS11 (SRSYGQQGSGS) [Seq. ID No. 67]	1 to 8	--
GAL4-V8x2 (DFDLDMLGDFDLDMLGSR) [Seq. ID No. 68]	1 to 12	++
GAL4-D (VP16 aa 459-490)	1 to 6	+++
GAL4-VP16 (VP16 aa 411-490)	1 to 4	++

III. Illustrative chimeric transcription factor for allostery-based systems

p65/GAL4/PR-LBD

An expression vector for directing the expression of an RU486 dependent transcription factor consisting of a progesterone receptor ligand binding domain, a GAL4 DNA binding domain and a p65 transcription activation domain can be prepared as follows. Primers 5'-p65-BglII and 3'-p65-BamHI can be used to amplify amino acids 361 to 550 of p65 from plasmid pCG-p65 (Rivera et al., Nature Medicine 2(9):1028-1032, 1996). The resulting fragment, which will have 5' BglII and 3' BamHI sites, can then be inserted into the BglII site of plasmid pGL (Wang et al, PNAS USA 91:8180-8184, 1994), which contains a truncated human progesterone receptor sequence (amino acids 640-891) and a GAL4 DNA binding domain sequence (amino acids 1-94). Up to 2 nucleotides may be added so that the p65 sequence is in frame downstream of the ATG and upstream of the GAL4 coding region.

5'-p65-BglII: agatctXGATGAGTTTCCCACCATG [Seq. ID No. 69]

3'-p65-BamHI: ggatccXGGAGCTGATCTGACTCAG [Seq. ID No. 70]

where X is 0, 1 or 2 nucleotides that may be required to create in-frame fusions.

ZFHD1/p65/PR-LBD

An additional RU 486 dependent transcription factor can be prepared using the composite DNA binding domain ZFHD1 (Rivera et al., supra, and US 08/366,083.) Primers 5'-PR-LBD and 3'-PR-LBD can be used to amplify amino acids 640-891 of hPRB891 from plasmid pT7bhPRB-891 (Vegeto et al, Cell 69:703-713, 1992). The resulting fragment, which will have 5' XbaI and 3' SpeI sites, can be inserted into the SpeI site of pCGNN-ZFHD1-p65. This will place the PR-LBD in-frame and at the carboxy terminus of p65.

5'-PR-LBD: 5'-tctagaAAAAAGTTCAATAAAGTCAG [Seq. ID No. 71]

3'-PR-LBD: 5'-actagtGCAGTACAGATGAAGTTG [Seq. ID No. 72]

rtTA/p65

A tetracycline inducible transcription factor containing the p65 activation domain can be constructed using pUHD17-1 (described in US 5,654,168) as follows. Digest pUHD17-1 with AflII. Remove the protruding 5' end with mung bean nuclease and ligate the synthetic oligonucleotide 5'-CactagtTAACTAAGTAA [Seq. ID No. 73]. The resulting plasmid, rTetR-SpeI contains a SpeI cleavage site at the very end of the rTetR gene.

Use primers 5'-p65-XbaI and 3'-p65-SpeI to amplify amino acids 361 to 550 of p65 from plasmid pCG-p65 (Rivera et al, supra) and clone this fragment, in frame, into the SpeI site of rTetR-SpeI.

5'-p65-XbaI: 5'-tctagaGATGAGTTTCCCACCATG [Seq. ID No. 74]

3'-p65-SpeI: 5'-actagtGGAGCTGATCTGACTCAG [Seq. ID No. 75]

IV. Regulated transcription mediated by the chimeric transcription factor S3H

The S3H activation domain was used to induce transcription of a SEAP target gene in response to rapamycin. S3H (p65(281-551)-HSF(406-530))- and S (p65(361-551)) -containing activation domain fusions were stably integrated into HT1080L cells (which already carry a stably integrated target SEAP gene) as part of the transcription factor vectors